Structure-proteasome-inhibitory activity relationships of dietary flavonoids in human cancer cells

Di Chen, Marina S. Chen, Qizhi Cindy Cui, Huanjie Yang, Q. Ping Dou

The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan, USA

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1. ABSTRACT

Diet high in vegetables and fruits has been associated with reduced cancer risk. However, the involved mechanisms are unknown. Previously, we reported that the dietary flavonoid apigenin could inhibit the proteasome activity and induce apoptosis in tumor cells. To further investigate the structure-proteasome-inhibitory activity relationships, we chose and tested five dietary flavonoids, including luteolin, apigenin, chrysin, naringenin and eriodictyol. We found that the order of inhibitory potencies and apoptosis-inducing potencies of these five compounds in 20S purified proteasome and tumor cells was: (1) luteolin > apigenin > chrysin, and (2) apigenin >> naringenin, and luteolin >> eriodictyol. Therefore, flavonoids with hydroxylized B ring and/or unsaturated C ring are natural potent proteasome inhibitors and tumor cell apoptosis inducers. Furthermore, neither apigenin nor luteolin could inhibit the proteasome and induce apoptosis in non-transformed human natural killer cells. This finding may provide a molecular basis for the clinically observed cancer-preventive effects of fruits and vegetables.

2. INTRODUCTION

Flavonoids are a group of polyphenolic substances widely distributed in plant kingdom and presented in human diets (1, 2). More and more studies suggest that consumption of diet high in fruits and vegetables lower the incidence risk of various cancers (1, 3-5). Plant-derived flavonoids may possess specific properties and bioactive effects that could benefit human health (6, 7).

The 26S proteasome is a multi-subunit protease complex and composed of the 20S core associated with two 19S regulatory caps (8, 9). Catalytic activities of the proteasome are located in the 20S core that has at least three major activities involved in target protein degradations: chymotrypsin-like, trypsin-like and caspase-like activities (10). The ubiquitin-proteasome pathway plays an important role in regulating both cell cycle and apoptosis (11-13). It has been shown that proteasome expression and activities are higher in tumor cells than a counterpart of normal cells, and that tumor cells are
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Figure 1. Chemical structures of five flavonoids.

Chemical structures of five flavonoids.

Dependent upon the proteasome function, as proteasome inhibition leads to growth arrest in the G1 phase of the cell cycle and/or induction of apoptosis (14, 15). Inhibition of the proteasome chymotrypsin-like activity is associated with induction of tumor cell apoptosis (16-20).

Previously we reported that some fruit and vegetable extracts and flavonoids (such as apigenin) could inhibit the proteasome activity and induce apoptosis in tumor cells (18, 19). In the current study, we investigated more natural and structurally related flavonoids commonly found in foods and plants for their structure-proteasome-inhibitory activity-relationships (SARs). These include chrysin (found in many plants, honey and propolis), luteolin (commonly found in broccoli, green chili, onion leaves, celery, pepper, olive oil and lemons), naringenin (found in orange and grapefruits), and eriodictyol (found in lemons) (21-24), with apigenin as a comparison. Chrysin, apigenin and luteolin possess identical A and C rings, but with different number of B-ring hydroxyl groups (0, 1 and 2, respectively; Figure 1). Apigenin and naringenin, as well as luteolin and eriodictyol, have identical A and B rings, with (unsaturated) and without (saturated) the 2,3-double bond in the C ring, respectively (Figure 1).

By performing an in vitro assay using a purified rabbit 20S proteasome, we found that the order of inhibitory potencies of these five flavonoids was: (1) luteolin > apigenin > chrysin, and (2) apigenin >> naringenin, and luteolin >> eriodictyol. We then investigated the proteasome-inhibitory and apoptosis-inducing abilities of these flavonoids in intact human leukemia Jurkat T and prostate cancer PC-3 cells. Again, the same ranks of potencies were obtained. Furthermore, the proteasome-inhibitory abilities of these flavonoids in Jurkat T and PC-3 cells correlated very well with their apoptosis-inducing potencies. Finally, neither apigenin nor luteolin could inhibit the proteasome activity and induce apoptosis in non-transformed human natural killer YT cells.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Chrysin [5,7-Dihydroxyflavone], apigenin [4', 5, 7-Trihydroxyflavone], luteolin [3',4',5,7-Tetrahydroxyflavone], naringenin [4',5,7-Trihydroxyflavanone], eriodictyol [(S)-3',4',5,7-Tetrahydroxyflavanone], protease inhibitor cocktail and dimethyl sulphoxide (DMSO) were purchased from Sigma–Aldrich Co. (Saint Louis, MO). Purified rabbit 20S proteasome, fluorogenic proteasomal chymotrypsin-like peptide substrate Suc-Leu-Leu-Val-Tyr-AMC, caspase-3 specific substrate Ac-Asp-Glu-Val-Asp-AMC, Protein G Plus/Protein A Agarose and Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (Z-LLL-CHO or MG132) were obtained from Calbiochem Inc. (San Diego, CA). Another fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity was from BIOMOL. Rabbit polyclonal antibody to Inhibitor of Nuclear Factor κB-α (IκB-α) and goat polyclonal antibody to actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal antibody to human poly(ADP-ribose) polymerase (PARP) was from BIOMOL. Mouse monoclonal IBA-11 antibody (16B12 clone) was purchased from Covance Research Products, Inc (Berkeley, CA). Fetal bovine serum (FBS) was obtained from Tissue Culture Biolbgicals (Tulare, CA). RPMI 1640 medium, penicillin, and
Figure 2. Flavonoids accumulate ubiquitinated proteins and a putative ubiquitinated form of IκB-α (p56) in Jurkat T cells. Jurkat T cells were treated with 50 µmol/L of the five flavonoids for indicated time points. The proteins of cell extracts were analyzed with SDS-PAGE gels and Western blot with ubiquitin (A), IκB-α (B) or actin antibodies. The p56 band of IκB-α was indicated by arrow.

streptomycin were purchased from Invitrogen Co. (Carlsbad, CA). X-tremeGENE Q2 Transfection Reagent was purchased from Roche Applied Science (Indianapolis, IN).

3.2. Cell culture, transfection and protein extract preparation

Human leukemia Jurkat T, prostate cancer PC-3 and non-transformed, immortalized human natural killer (YT) cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. All the cell lines were maintained at 37° C in a humidified incubator with an atmosphere of 5% CO₂. X-tremeGENE Q2 Transfection Reagent was used for transfection according to the manufacturer’s instructions. The hemagglutinin (HA)-tagged poly-ubiquitin expressing plasmid, pMT123 (HA-Ub), was kindly provided by Dr. Y. Haupt (Hebrew University, Jerusalem, Israel) and used for transfection (4 µg DNA per 1 x 10⁶ Jurkat cells). A whole cell extract was prepared as described previously (25), and the extracts obtained from the transfected cells were also used for preparation of immunoprecipitates (see below).

3.3. Inhibition of purified 20S proteasome activity by flavonoids

The chymotrypsin-like activity of purified 20S proteasome was measured as described previously (19). Briefly, 35 ng of purified 20S proteasome was incubated in 100 µl of assay buffer (50 mmol/L Tris–HCl, pH 7.5) with or without different concentrations of each flavonoid and
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Figure 3. Accumulation of ubiquitinated forms of IκB-α protein was verified by IP-Western analysis. Jurkat T cells were transiently transfected with the pMT123 (HA-Ub) or mock transfection (lane 4) for 48 h. The cells were then treated with indicated concentration of apigenin (lanes 1 to 3) or 5 µmol/L of MG132 (lane 5) for 24 h. As a control, the untransfected cells were treated with 5 µmol/L of MG132 (lane 4). Lysates from transfected cells were incubated with rabbit polyclonal antibody against IκB-α and protein A/G affinity beads (lanes 2 to 5) or rabbit IgG and protein A/G affinity beads as control (lane 1). The samples were processed as described in Material and Methods. The transferred membrane was immunoblotted with HA-11 antibody (lanes 1 to 5). Bands of ubiquitinated IκB-α proteins (p100, p56) were indicated.

20 µmol/L fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) for 2 h at 37° C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3TM multilabel counter with an excitation filter of 355 nm and an emission filter of 460 nm.

3.4. Caspase-3 activity assay

Caspase-3 activities were determined by measuring the release of the AMC groups from a caspase-3 specific substrate Ac-Asp-Glu-Val-Asp-AMC. Briefly, Jurkat T or PC-3 cells were treated with 50 µmol/L of each flavonoid for different time points, followed by preparation of whole cell extracts. The cell extract (20 µg) was then incubated with 20 µmol/L of the caspase-3 substrate in 100 µl of the assay buffer (50 mmol/L Tris-HCl, pH 7.5) in a 96-well plate. The reaction mixture was incubated at 37° C for 3 h and the hydrolyzed fluorescent AMC groups were quantified as described above.

3.5. Inhibition of the proteasome activity in intact tumor cells by flavonoids

To measure the inhibition of proteasome activity in living tumor cells, 100 µl of Jurkat T or PC-3 cells (1 × 10⁴ cells/well) were cultured in a 96-well plate. These cells were treated with or without various concentrations of flavonoids for 24 h, followed by an additional incubation for 2 h with the fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured using the same plate reader and conditions mentioned above.

3.6. Western blot analysis

Jurkat T or YT cells were treated with flavonoids for indicated hours (see figure legends), followed by preparation of whole cell extracts. Equal amount of protein extract (30 µg) from each sample was then separated by an SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane. The enhanced chemiluminescence (ECL) Western blot analysis was performed using specific antibodies against IκB-α, PARP or actin.

3.7. Immunoprecipitation (IP)-Western assay

Cell lysates prepared from the plasmid or mock-transfected Jurkat cells were precleared with 50 µl of Protein G/Protein A agarose beads for 1 h at 4° C. The supernatants were transferred to fresh Eppendorf tubes, and 6 µg of rabbit polyclone IκB-α antibody was added to each sample. Normal rabbit IgG was used as control. The mixtures were incubated for 2 h at 4° C and then 50 µl of the beads was added for overnight mixing at 4 °C. After that, the beads were washed and boiled in SDS-PAGE sample buffer, followed by SDS-PAGE gel analysis and Western blotting with mouse monoclonal antibody against HA.

4. RESULTS

4.1. Structural relationships of several dietary flavonoids to their activities to inhibit purified 20S proteasome

Previously we reported that the dietary flavonoid apigenin is a natural inhibitor of the chymotrypsin-like activity of a purified 20S proteasome and tumor cellular 26S proteasome (19). To further study the structural relationships of apigenin to its proteasome-inhibitory activity, in the current study we examined several related flavonoids.

We first examined the role of number of OH groups on the B-ring of apigenin. To do so, chrysin, apigenin and luteolin were selected, all of which have identical A- and C-ring structures, but with none, one or two OH groups connected to the B-ring, respectively (Figure 1). A cell-free proteasome activity assay was performed by incubating a purified rabbit 20S proteasome and a specific chymotrypsin-like substrate in the presence of each of these three flavonoids at different concentrations. We determined the IC₅₀ values of chrysin, apigenin and luteolin as 4.9, 2.3 and 1.5 µmol/L,
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Table 1. IC<sub>50</sub> values of the flavonoids for inhibition of the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in intact Jurkat T cells<sup>1</sup>

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (in 20S) (µmol/L)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (in 26S) (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysin</td>
<td>4.9 (±0.15)</td>
<td>6.1 (±0.44)</td>
</tr>
<tr>
<td>Apigenin</td>
<td>2.3 (±0.20)</td>
<td>1.9 (±0.17)</td>
</tr>
<tr>
<td>Luteolin</td>
<td>1.5 (±0.10)</td>
<td>1.3 (±0.11)</td>
</tr>
<tr>
<td>Naringenin</td>
<td>48.9 (±1.96)</td>
<td>41.6 (±1.47)</td>
</tr>
<tr>
<td>Eriodictyol</td>
<td>16.2 (±0.62)</td>
<td>14.6 (±0.95)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Inhibition of the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in intact Jurkat T cells was measured as described in “Materials and methods”.

respectively (Table 1), suggesting that the OH groups on the B-ring might be important for inhibiting the proteasomal chymotrypsin-like activity (see Discussion).

We noticed that another dietary flavonoid, naringenin possesses almost identical structure to that of apigenin, except that naringenin lacks the 2,3-double bond of apigenin (Figure 1). In addition, the flavonoid eriodictyol has the identical structure of luteolin without the 2,3-double bond (Figure 1). We took advantage of this difference between apigenin and naringenin as well as luteolin and eriodictyol to investigate the potential role of this double bond in inhibiting the proteasomal chymotrypsin-like activity. By performing the same cell-free activity assay using the purified 20S proteasome, IC<sub>50</sub> values of naringenin and eriodictyol were determined. Comparing to the potent counterpart apigenin (IC<sub>50</sub> 2.3 µmol/L), naringenin was much less potent with an IC<sub>50</sub> value of 48.9 µmol/L (Table 1). Consistently, removal of the double bond in luteolin (IC<sub>50</sub> 1.5 µmol/L) produces a much less active analog eriodictyol (IC<sub>50</sub> 16.2 µmol/L; Table 1). Therefore, the 2,3-double bond in the C-ring is essential for proteasome-inhibitory activity of these dietary flavonoids.

4.2. Structural relationships of dietary flavonoids to their activities to inhibit tumor cellular proteasome

To determine whether these flavonoids could also inhibit the activity of 26S proteasome in living tumor cells according to the identified SARs (Figure 1, Table 1), human leukemia Jurkat T cells were treated with each of these five flavonoids at various concentrations, followed by an additional incubation with a fluorogenic proteasome peptide substrate specifically for the proteasomal chymotrypsin-like activity. Afterwards, cells were measured for levels of hydrolyzed AMC groups (see Materials and Methods). The results from this cellular proteasome study (Table 1, 26S) were similar to those generated with purified 20S proteasome (Table 1, in 20S). The order of potencies to inhibit cellular proteasome activity was found to be: luteolin (IC<sub>50</sub> 1.3 µmol/L) > apigenin (IC<sub>50</sub> 1.9 µmol/L) > chrysin (IC<sub>50</sub> 6.1 µmol/L) (Table 1, 26S), supporting the idea that the B-ring OH groups play an important role in proteasome inhibition. Also, the flavonoids with the 2,3-double bond in their C-ring, apigenin (IC<sub>50</sub> 1.9 µmol/L) and luteolin (IC<sub>50</sub> 1.3 µmol/L) showed much more potent inhibitory effects on proteasomal chymotrypsin-like activity in intact Jurkat T cells than their analogs without the double bond, naringenin (IC<sub>50</sub> 41.6 µmol/L) and eriodictyol (IC<sub>50</sub> 14.6 µmol/L), respectively (Table 1, 26S). These data are consistent with the role of the 2,3-double bond of flavonoids in inhibiting the proteasome activity.

To verify their proteasome-inhibitory effects in cultured tumor cells, we determined whether these flavonoids could have an effect on accumulation of ubiquitinated proteins and natural proteasome target proteins, such as IκB-α, in intact tumor cells. Jurkat T cells were treated with 50 µmol/L of each flavonoid for up to 12 h, followed by preparation of protein extracts and measurement of ubiquitinated proteins in Western blot analysis (Figure 2A). The order of accumulated ubiquitinated proteins, such as at 12 h, was: luteolin > apigenin >> chrysin; apigenin >>> naringenin; luteolin >>> eriodictyol, consistent with the SARs found using purified 20S proteasome and in cultured tumor cells (Table 1).

Previously we have reported that apigenin and quercetin were able to accumulate a candidate ubiquitinated form of IκB-α with 56 kDa (p56) (19). The level of this p56 band, detectable by the specific antibody to IκB-α, was also increased in Jurkat T cells treated with luteolin, apigenin and chrysin in time-dependent manner (indicated by an arrow, Figure 2B). The order of increased p56 levels at 24 h was: luteolin, apigenin > chrysin. When the cells treated with naringenin or eriodictyol were examined and compared, little or no increase in p56 protein expression was observed (Figure 2B). Again, the potency order was: apigenin >>> naringenin, and luteolin >>> eriodictyol, consistent with the established SARs (Table 1).

To confirm that p56 is an ubiquitinated form of IκB-α protein, we performed an immunoprecipitation-Western blot assay using protein extracts prepared from cells transfected with HA-tagged ubiquitin-expressing plasmid. Briefly, Jurkat cells were transiently transfected with the HA-tagged ubiquitin-expressing plasmid pMT123 for 48 h and then treated with apigenin (at 25 or 50 µmol/L) or the authentic proteasome inhibitor MG132 (as a positive control, at 5 µmol/L) for 24 h. The prepared cell extracts were immunoprecipitated with a specific IκB-α antibody complexed with protein A/G agarose, and the IκB-α immunoprecipitates were then blotted with an anti-HA antibody. The results (Figure 3) showed that a similar p56 band was detected in cells transfected with HA-ubiquitin and treated with 50 µmol/L apigenin (Figure 3, lane 2). The p56 was also increased in a less abundant
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Figure 4. Flavonoids induce cell death and apoptosis associated with their potencies of proteasome inhibition. Jurkat T cells were treated with indicated concentrations of the five flavonoids for 24 h, followed by Trypan blue dye exclusion assay (A) or treated with 50 µmol/L of the flavonoids for different time points, followed by lyses of cells and preparing whole cell extracts that were used for caspase-3 activity assay (B) and Western blotting with PARP antibody (C). DMSO (DM) was used as a solvent control in all experiments above. The experiments were repeated three times with the similar results.

amount in cells transfected with HA-ubiquitin and treated with 5 µmol/L MG132 (Figure 3, lane 5). Surprisingly, a band of ~100 kDa (p100) was found in HA-ubiquitin-transfected Jurkat T cells after treatment with apigenin (Figure 3, lanes 2, 3). The induction levels of this p100 were dependent on the apigenin concentrations used (Figure 3, lanes 2, 3). This p100 is related to IκB-α, since this protein was not detected in the preparation where the IκB-α antibody was eliminated in the immunoprecipitate step (using normal rabbit IgG and protein A/G agarose) (Figure 3, lanes 1 vs. 2). As a positive control, p100 was also significantly increased in cells transfected with HA-ubiquitin and treated with MG132 (Figure 3, lane 5). This p100 is also related to ubiquitin, since this protein was not detected in mock-transfected cells treated with MG132 (Figure 3, lanes 4 vs. 5). Therefore, the dietary flavonoids such as apigenin indeed act as natural proteasome inhibitors in intact tumor cells.

4.3. Structural relationships of dietary flavonoids to their activities to induce apoptotic cell death

It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of tumor apoptotic cell death (16, 19, 20). We then investigated the cell death-inducing potencies of these five flavonoids. Jurkat T cells were treated with 5, 25, or 50 µmol/L of each flavonoid for 24 h, and then analyzed with the Trypan blue dye exclusion assay to determine the extent of cell death (Figure 4A). A dose-dependent cell death was observed when each of these flavonoids was used. At 25 µmol/L, chrysin, apigenin and luteolin induced 30%, 45% and 60% cell death, respectively (Figure 4A),
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Figure 5. Proteasome inhibition and caspases activation by flavonoids in human prostate cancer PC-3 cells. PC-3 cells were treated with 50 µM/L of each of the five flavonoids for indicated time points, DMSO (DM) was used as solvent control, followed by measure the proteasomal chymotrypsin-like (CT-like) (A) and caspase-3 activities (B) in the cell extracts.

While naringenin and eriodictyol resulted in 20% cell death or less (Figure 4A). At 50 µmol/L, chrysin, apigenin and luteolin resulted in 60%, 82% and 86% nonviable cells, respectively (Figure 4A), while naringenin and eriodictyol resulted in 20% and 30% cell death, respectively (Figure 4A).

To confirm that the cell death induced by these flavonoids is apoptosis, we measured their apoptosis-inducing activities by caspase-3 activation and PARP cleavage in Jurkat T cells treated with each flavonoid for up to 12 h (see Figure 4B). The caspase-3 activities start to increase at as early as 2 h treatment. The fold of increased caspase-3 activity at 12 h is: luteolin, 11.0 > apigenin, 9.7 > chrysin, 8.4 > eriodictyol, 3.7 > naringenin, 2.7 (Figure 4B), consistent with the results of cell death (Figure 4A) and proteasome inhibition (Table 1 and Figure 2A).

Caspase-3 is an essential effector caspase responsible for cleaving PARP in many cell systems. We then measured PARP cleavage. Chrysin, apigenin and luteolin induced apoptosis-specific PARP cleavage to p85 at as early as 2 h (Figure 4C). At 12 h, almost all the PARP was cleaved in cells treated with apigenin and luteolin, but not chrysin (Figure 4C). In contrast, very low levels of the cleaved PARP were induced by 50 µmol/L of eriodictyol, and no PARP cleavage was found after treatment with 50 µmol/L naringenin (Figure 4C). The data confirm that dietary flavonoids act as proteasome inhibitors and apoptosis inducers in tumor cells and that their biological activities require the presence of OH groups on the B-ring and 2,3 double bond in the C-ring.

4.4. The dietary flavonoids can also inhibit proteasome activity and induce caspase-3 activation in human prostate cancer PC-3 cells

To confirm whether the selected flavonoids have the similar effects in human solid tumor cells, we treated human prostate cancer PC-3 cells with 50 µmol/L of each of the five flavonoids for 6, 12 and 24 hours, followed by measuring levels of the proteasomal chymotrypsin-like and caspase-3 activities. We found that after 24 h-treatment, the proteasome chymotrypsin-like activity in PC-3 cells treated with luteolin, apigenin and chrysin was inhibited by 58.5, 50.3 and 47.2%, respectively (Figure 5A). However, there were little inhibitory effects observed in the cells exposed to naringenin or eriodictyol (Figure 5A). Proteasome inhibition caused PC-3 cell apoptosis, as evident by caspase activation. The levels of caspase-3 activities were increased by 3.0-, 2.8- and 2.3-fold, respectively, by treatment of luteolin, apigenin and chrysin (Figure 5B). In contrast, neither naringenin nor eriodictyol had much effect (Figure 5B). Therefore, the dietary flavonoids with B-ring OH groups and C-ring 2,3 double
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Figure 6. Apigenin and luteolin selectively inhibits proteasome activity and induce apoptosis in leukemia Jurkat T cells but not in normal YT cells. The Jurkat T and YT cells were treated with various concentrations of apigenin or luteolin or DMSO (as a control) for 24 h, followed by determining PARP cleavage (A) and treated for 12 h for proteasomal chymotrypsin-like activity (B).

bond are proteasome inhibitors and apoptosis inducers in human prostate cancer cells.

4.5. Non-transformed human natural killer cells are more resistant to apigenin and luteolin than human leukemia cells

We have shown that flavonoids such as apigenin and luteolin can inhibit the proteasome activity and induce tumor cell apoptosis (Figures 2, 4 and 5). However, whether these potent flavonoids could affect on human normal or non-transformed cells was unknown. To determine whether apigenin and luteolin were able to induce apoptosis preferentially in tumor/transformed vs. normal/non-transformed cells, we treated both human leukemia Jurkat T cells and immortalized, non-transformed natural killer cells (YT cell line) with apigenin and luteolin at various concentrations for 24 h (Figure 6). Reproducibly, apigenin and luteolin at 25-50 µmol/L induced apoptosis-specific PARP cleavage in Jurkat T cells (Figure 6A). In sharp contrast, no PARP cleavage was detectable in the YT cells after treatment with either apigenin or luteolin under identical conditions (Figure 6A).

To investigate whether apigenin and luteolin have differential effects on the proteasomal activity of Jurkat T vs. YT cells, both cell lines were treated with apigenin and luteolin at 1, 25 or 50 µmol/L for 12 h, followed by an additional incubation for 2 h with a fluorogenic peptide substrate specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured (Figure 6B). In Jurkat T cells, treatment with apigenin or luteolin caused a concentration-dependent inhibition of the proteasomal chymotrypsin-like activity with >90% inhibition at 50 µmol/L (Figure 6B). In contrast, the proteasomal chymotrypsin-like activity in YT cells was decreased by only ~15% with apigenin or luteolin at the highest concentration used (Figure 6B). Therefore, the proteasome activity in non-transformed YT cells is only slightly inhibited by apigenin and luteolin, which may be responsible for lack of apoptosis in these cells (Figure 6A).
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5. DISCUSSION

An estimated 6.2 million people worldwide are die annually from the cancers (26, 27). The cancer chemoprevention is an important strategy to decrease the incidence of cancers and related mortality. Many studies have shown that diet high in vegetables and fruits is associated with reducing the risk of cancer (3, 4). Flavonoids are polyphenolic compounds and rich in variety of vegetables, fruits and beverages such as tea, cocoa and wine (28, 29). Previously we reported that grape and apple extracts as well as their dietary flavonoids, apigenin and quercetin, could inhibit proteasome activity in human cancer cells and induce apoptotic cell death (18, 19). The purpose of current study is to discover more effective and potent dietary flavonoids as proteasome inhibitors. We found that chrysin, luteolin, naringenin and eriodictyol have similar structures to that of apigenin. We hypothesized that these flavonoids might have altered proteasome-inhibitory and biological effects.

By determining the inhibitory activities of chrysin, apigenin and luteolin and then comparing their chemical structures, the first SAR was identified, which is that the OH groups on the B-ring might be important for inhibiting the proteasomal chymotrypsin-like activity (Figure 1 and Table 1). The potency of proteasomal inhibition was increased along with increase of the number of OH groups on their B-ring from none to two (Figure 1 and Table 1). Consistently, we also noticed that kaempferol and quercetin have identical A and C rings, but with one or two B-ring OH groups, respectively and that quercetin, could inhibit proteasome activity in human cells and induce apoptotic cell death (18, 19). The second SAR identified in the current study was that the 2,3-double bond of flavonoids is required for potently inhibiting the proteasome activity. According to the chemical structure, we divided the selected flavonoids into two groups. Chrysin, apigenin and luteolin all have unsaturated bonds in their C ring, called “unsaturated C-ring group”. Naringenin and eriodictyol contain “saturated C-ring group” since they all have single bond in the C-ring (Figure 1). We found that flavonoids in unsaturated C-ring group were much more potent (IC_{50} from 1.3 to 6.1 µmol/L) than those in saturated C-ring group (IC_{50} from 14.6 to 41.6 µmol/L) (Table 1, 26S). The kinetic experiment using Jurkat T cells treated with these flavonoids showed differential levels of ubiquitinated proteins and an ubiquitinated form of the proteasome target IκB-α (Figure 2), supporting the rank of the proteasomal-inhibitory activities of these flavonoids. Increased levels of ubiquitinated forms of the proteasome target IκB-α by apigenin were verified by the data from immunoprecipitation-Western blot assay (Figure 3).

To further verify that these flavonoids can inhibit proteasomal activity in intact human cancer cells, we treated the Jurkat T cells with these five flavonoids in different concentrations and performed proteasomal activity assay. Consistent with their in vitro potencies, flavonoids in unsaturated C-ring group were much more potent (IC_{50} from 1.3 to 6.1 µmol/L) than those in saturated C-ring group (IC_{50} from 14.6 to 41.6 µmol/L) (Table 1, 26S). The orders of potency of cellular proteasomal inhibition and cell death induction are: (1) luteolin > apigenin > chrysin, and (2) apigenin >> naringenin, and luteolin >> eriodictyol (Table 1, 26S, and Figures 2, 4), the two SARs identified using the purified rabbit 20S proteasome (Table 1, 20S). To confirm the observation in solid tumor cells we tested the flavonoids in human prostate cancer PC-3 cells (Figure 5). The results were consistent with the finding in leukemia Jurkat T cells. These data demonstrate that the selected flavonoids have the similar biological effects in various human tumor cell lines. However, we found that human solid tumor cells are more resistant to treatment of the selected flavonoids than leukemia cells.

It is known that some of anticancer drugs act through their cellular toxicity without specifically killing tumor over normal cells. It has been shown that proteasome expression and activities are higher in tumor cells compared with a counterpart of normal cells, and that survival of tumor cells are dependent on the proteasome function (14, 15). We hypothesize that some selected flavonoids like those studied here are natural proteasome inhibitors, which could preferentially inhibit tumor cellular.
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proteasome activity (which is higher than normal cells) and induce apoptotic cell death. Consistently, our results showed that there was no induction of apoptosis in non-transformed human natural killer YT cells treated with apigenin and luteolin when compared to human leukemia Jurkat T cells (Figure 6A) and that only around 15% of the proteasomal chymotrypsin-like activity in YT cells was inhibited by 50 μmol/L of apigenin and luteolin treatment, in contrast to around 90% inhibition in Jurkat T cells (Figure 6B). These data support the idea that luteolin, apigenin and chrysin have great potential to be developed into novel cancer preventative agents that function through the mechanism of proteasome inhibition selectively in cancer over normal cells.

In conclusion, some flavonoids, such as luteolin, apigenin and chrysin, are natural proteasome inhibitors and inhibition of tumor cellular proteasome by these flavonoids should selectively trigger cancer cell apoptosis. The results here demonstrate that proteasome inhibition may contribute to the cancer-preventive effects of these flavonoids and also help describe the structure-activity relationships among these compounds.

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7. REFERENCES

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**Send correspondence to:** Q. Ping Dou, Ph.D., The Prevention Program, Barbara Ann Karmanos Cancer Institute and Department of Pathology, School of Medicine, Wayne State University, 516 HWCRC, 4100 John R, Detroit, Michigan, 48201, U.S.A., Tel: 313-576-8301, Fax: 313-576-8307, E-mail: doup@karmanos.org

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